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<p>(54) Title: HIGHLY CONCENTRATED, LYOPHILIZED, AND LIQUID FACTOR IX FORMULATIONS (57) Abstract Provided by the present invention are novel compositions and methods for obtaining highly concentrated, liquid, and lyophilized preparations of factor IX suitable for storage and administration.</p>		

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HIGHLY CONCENTRATED, LYOPHILIZED, AND LIQUID FACTOR IX FORMULATIONS

5

FIELD OF INVENTION

The present invention relates generally to novel formulations comprising factor IX, including both highly concentrated, lyophilized, and liquid formulations comprising factor IX suitable for administration via various routes including for example routes such as *intravenous*,
10 *subcutaneous*, *intramuscular* and *intra dermal*.

BACKGROUND OF THE INVENTION

A variety of factors involved in the blood clotting process have been identified, including factor IX, a plasma glycoprotein. A deficiency of factor IX characterizes a type of hemophilia (type B). Treatment of this disease has traditionally involved *intra venous* infusion
15 of human plasma-derived protein concentrates of factor IX. Infusion of blood concentrates involves the risk of transmission of various infectious agents, such as viral hepatitis and HIV, or thromboembolic factors. An alternative method of producing factor IX, by recombinant DNA techniques, has been described in USPN 4,770,999, Kaufman *et al.*, September 13, 1988. The cDNA coding for human factor IX has been isolated, characterized, and cloned into
20 expression vectors. See, for example, Choo *et al.*, Nature 299:178-180 (1982); Fair *et al.*, Blood 64:194-204 (1984); and Kurachi *et al.*, Proc. Nat. Acad. Sci., U.S.A. 79:6461-6464 (1982). Thus, through advances in recombinant DNA technology, it has been possible to produce factor IX protein.

25 It is desirable to have both bulk and finished forms of factor IX, suitable for both storage and for delivery. Typically, a purification process for a protein results in concentrating the protein. This concentrated protein, also known as bulk protein, may be in a formulation buffer. Bulk protein, typically at a concentration of about 2 to at least 20 mg/mL, can then be shipped frozen to a fill/finish facility where it is adjusted to an appropriate dosage
30 concentration and placed into dosage vials or some device suitable for administration, *e.g.* a pre-fillable syringe. Ideally, the drug product is left in the liquid state and stored and administered as a liquid. Alternatively, the drug product is lyophilized, *i.e.*, freeze-dried. Ideally lyophilized drug product has sufficient stability to be kept in long-term storage, *i.e.*, greater than six months; lyophilized drug product is reconstituted at a later time by adding a
35 suitable administration diluent just prior to patient use.

The decision to either maintain the finished drug product as a liquid or to freeze-dry it is usually based on the stability of the protein drug in those forms. Protein stability can be affected *inter alia* by such factors as ionic strength, pH, temperature, repeated cycles of freeze/thaw, and exposures to shear forces. Active protein may be lost as a result of physical instabilities, including denaturation and aggregation (both soluble and insoluble aggregate formation), as well as chemical instabilities, including, for example, hydrolysis, deamidation, and oxidation, to name just a few. For a general review of stability of protein pharmaceuticals, see, for example, Manning, *et al.*, *Pharmaceutical Research* 6:903-918 (1989).

While the possible occurrence of protein instabilities is widely appreciated, it is impossible to predict particular instability problems of a particular protein. Any of these instabilities can result in the formation of a protein, protein by-product, or derivative having lowered activity, increased toxicity, and/or increased immunogenicity. Indeed, protein precipitation may lead to thrombosis, non-homogeneity of dosage form and amount, as well as clogged syringes. Also, specific to factor IX, there are several post-translational modifications (for example, the gamma carboxylation of certain glutamic acid residues in the N-terminus and the addition of carbohydrate) all of which provide potential sites that may be susceptible to modification upon storage. Thus, the safety and efficacy of any pharmaceutical formulation of a protein is directly related to its stability. Maintaining that stability in a liquid dosage form is generally different from a lyophilized dosage form because of greatly increased potential for molecular motion and therefore increased probability of molecular interactions. Maintaining stability in a highly concentrated form is also different because of the propensity for aggregate formation at high protein concentrations.

When developing a liquid formulation, many factors are taken into consideration. Short-term, *i.e.*, less than six months, liquid stability generally depends on avoiding gross structural changes, such as denaturation and aggregation. These processes are described in the literature for a number of proteins, and many examples of stabilizing agents exist ("Strategies to Suppress Aggregation of Recombinant Keratinocyte Growth Factor during Liquid Formulation Development", B.L. Chen *et al.*, *J. Pharm. Sci.* 83(12):1657-1661, (1994); "Formulation Design of Acidic Fibroblast Growth Factor", P.K. Tsai *et al.*, *Pharm. Res.* 10(5):649-659 (1993); "The Stabilization of Beta-Lactoglobulin by Glycine and NaCl", Tsutomu Arakawa, *Biopolymers* 28:1397-1401 (1989); "Structural stability of lipase from wheat germ", A.N. Rajeshwara and V. Prakash, *Internat. J. of Peptide & Prot. Res.* 44:435-440 (1994); "Thermal Stability of Human Immunoglobulins with Sorbitol", M. Gonzalez *et al.*, *Vox Sang* 68:1-4 (1995)). It is well known that an agent effective at stabilizing one protein

actually acts to destabilize another. Once the protein has been stabilized against gross structural changes, developing a liquid formulation for long-term stability (greater than six months, for example) depends on further stabilizing the protein from types of degradation specific to that protein. More specific types of degradation may include, for example, disulfide bond
5 scrambling, oxidation of oligosaccharides and/or certain residues, deamidation, cyclization, and the like. Although it is not always possible to pinpoint the individual degradation species, assays are developed to monitor subtle changes so as to monitor the ability of specific excipients to uniquely stabilize the protein of interest.

In addition to stability considerations, one generally selects excipients which will meet
10 with the approval of various world-wide medical regulatory agencies. It is highly desirable that the formulation be approximately isotonic and that the pH of the formulation be in a physiologically suitable range upon injection/infusion, otherwise pain and discomfort for the patient may result. The choice and amount of buffer used is important to achieve the desired pH range. The choice and amount of agents used to modify tonicity is important to assure ease
15 of administration.

Traditionally, large labile proteins, such as factor IX, are administered *intravenous*, either prophylactically or in response to bleeding episodes. Given *intravenous*, the protein is directly available in the blood stream. Unfortunately, there can be side effects associated with repeated injections, including occlusion and/or fibrin formation, especially in the elderly.
20 Moreover, where the patient's veins are particularly small, *e.g.*, in small children, it can be difficult to achieve the requisite therapeutic dose.

Currently, there are no highly concentrated factor IX formulations commercially available. The only two commercially available (in the US), carrier-protein-free, plasma-derived factor IX formulations, are freeze-dried products which are reconstituted for use, and
25 are limited to low factor IX concentrations, *e.g.*, about 100 U/mL or less than 1 mg/mL. Such low concentrations are primarily indicated for *intravenous* administration and not intended for *subcutaneous, intramuscular, or intradermal* use. Alpha Therapeutic Corporation provides lyophilized AlphaNine® SD, comprising heparin, dextrose, polysorbate 80, and tri(n-butyl) phosphate. This preparation is meant to be stored at temperatures between 2° and 8°C.
30 Heparin is to be avoided as it is an anti-coagulant and tri(n-butyl) phosphate is irritating to mucous membranes; thus, this formulation is less than ideal. Armour Pharmaceutical Company provides lyophilized Mononine®, comprising histidine, sodium chloride and mannitol, is similarly meant to be stored at 2° to 8°C. The package insert recommends not storing this formulation for greater than one month at room temperature. There are no liquid nor any

highly concentrated factor IX products currently commercially available. Schwinn, PCT/EP90/02238, discloses factor IX, 0.9 M saccharose, 0.5 M lysine, and 0.003 M calcium chloride, stored at 4-8°C, stable for only a period of weeks and therefore, unsuitable for commercial production; this formulation is, unfortunately hypertonic and the pH is outside the range for comfortable administration, and therefore unsuitable for injection.

Easier to handle for the patient are administration forms such as *subcutaneous*, *intramuscular*, or *intra-dermal*. *Subcutaneous* administration of factor IX is described in Berrettini, Am. J. Hematol. 47:61 (1994) and in WO 93/O7890, BTG, Brownlee (published 29 April 1993). In Berrettini, an Immuno product was used: Immunine™; factor IX, heparin, sodium citrate, sodium chloride, and antithrombin III at a concentration of 118 U/mg. The product was reportedly poorly and slowly transported into the circulation and the authors concluded that *subcutaneous* administration was not reliable for treating or preventing bleeding in hemophilia B patients and that even more concentrated forms would be unacceptable in terms of clinical efficacy. Brownlee, *supra*, discloses a Mononine™ factor IX formulation at a concentration of 10-500 U/mL. Only low circulating levels were obtained and at page 9, it is noted that after four hours large clots had formed under the skin at each site where the factor IX had been injected resulting in severe bruising. Such can be observed when using a product that is impure.

In hemophilia B dogs (Brinkhous, *et al.*, FASEB 7:117 (1993)) and in one hemophilia B patient (Liles, *et al.*, Thromb. Haemost. 73:1986a (1995)), plasma derived factor IX (pFIX) was administered *subcutaneously* (at doses of 15-47 U/kg in dogs and at a dose of 30 U/kg in the patient). This resulted in plasma factor IX in dogs which was dose dependent and ranged from 0.8 to 7.6%, with *intramuscular* giving higher levels. In the hemophilia patient, plasma factor IX activity reached only 1% within six hours; this level of activity persisted for 36 hours; the low concentration of plasma-derived factor IX required high volume injections at multiple (10) sites.

One of the major problems associated with formulating a suitable *subcutaneous* formulation is achieving a high enough concentration of the protein, without causing aggregate formation of the protein and without simultaneously concentrating impurities in the preparation. Both aggregate formation and impurities lead to increased immunogenicity. With currently available products, to give an appropriate dose of factor IX *subcutaneously* requires the use of multiple injection sites. This causes great discomfort and inconvenience to the patient. To

practically deliver factor IX *subcutaneously*, it is necessary to concentrate factor IX to at least 1,000 U/mL or greater and provide it in a stable, non-aggregating dosage form. Such a concentrated form is currently unavailable.

5 Ideally, formulations should provide for factor IX stability for greater than one year and for compatibility over a wide range of protein concentration (0.1 mg/mL to greater than 160 mg/mL, *i.e.*, 20 U/mL to greater than 56,000 U/mL for example). This allows for flexibility in methods of administration which may require high protein concentration, *e.g.*, *subcutaneous*, *intradermal*, or *intramuscular* administration, or those which may utilize low protein concentration, *e.g.* *intra venous* administration. Generally, more highly concentrated forms
10 allow for the administration of lower volumes which is highly desirable from the patients' point of view. Liquid formulations can have many advantages over freeze-dried products with regard to ease of administration and use. Accordingly, there continues to exist a need in the art for methods for improving factor IX protein stability, increasing the concentration, maintaining activity levels, and providing stable liquid formulations suitable for prolonged storage for
15 greater than one year at 2 to 8°C.

BRIEF SUMMARY OF THE INVENTION

One aspect of the present invention provides novel compositions and methods for providing highly concentrated, lyophilized, and liquid preparations of factor IX useful as bulk
20 protein or useful for administration. These compositions, either frozen or liquid, are stable for at least six months, and preferably up to 36 and 60 months; and can be stored at temperatures ranging from -100°C to 40°C, from -80°C to 0°C, and from -20°C to 10°C. The compositions comprise factor IX, tonicity modifiers, cryoprotectants, and, optionally, a buffering agent and/or other excipients which further stabilize factor IX. The factor IX concentration ranges
25 from about 0.1 to about 160 mg/mL (equivalent to about 20 to at least 56,000 U/mL), with 1 to 160 mg/mL (250 to 56,000 U/mL) and 0.1 to 10 mg/mL (25 to 2500 U/mL) preferred, the most preferred range depending upon the route of administration. Tonicity modifiers include, but are not limited to, salts, sugars, polyols, and amino acids. Suitable amino acids include arginine, glycine and histidine at a concentration of about 10 to 500 mM, with about 10 to 300
30 mM and about 10 to 200 mM preferred. Suitable cryoprotectants include polyols, *e.g.* mannitol and sucrose, and range in concentration from about 1 to 400 mM, with about 5 to 200 mM and 20 to 100 mM preferred. Optionally, the compositions may also contain a surfactant or detergent, such as polysorbate (*e.g.* Tween) or polyethyleneglycol (PEG), which may also serve as a cryoprotectant during freezing. The surfactant ranges from about 0.005 to 1%, with

about 0.005 to 0.1% and about 0.005 to 0.02% preferred. Optionally, the composition may contain an appropriate buffering agent to maintain a physiologically suitable pH, *e.g.*, in the range of about 5.8 to 8.0 with about 6.2 to 7.2 and about 6.5 to 7.0 being preferred. Buffering agents preferably include histidine, sodium citrate, potassium citrate, maleic acid, ammonium acetate, Tris, sodium phosphate, potassium phosphate, and diethanolamine, with sodium/potassium citrate preferred, with a preferred pH of about 6.5 to 7.5, and a concentration range of about 1-100 mM, with 5 to 50 mM and 10 to 25 mM preferred. Optionally, small amounts of a chelator such as EDTA are included, at a concentration of 0.05 to 50 mM, or 0.05 to 10 mM, or 0.1 to 5 mM, with about 1 to 5 mM preferred.

Another aspect of the present invention provides formulations of factor IX suitable for administration in a final dosage form, for example, via *intravenous*, *subcutaneous*, *intra-dermal*, or *intramuscular* routes of administration. Typically, large quantities of bulk drug are frozen and can be shipped, if necessary, to a manufacturing site where the bulk drug is filled into small vials; if desired, the final dosage form is a diluted, pH-adjusted form; bulk drug typically comprises a higher protein concentration than finished drug and does not need to be isotonic. The finished drug compositions comprise factor IX, tonicity modifiers, cryoprotectants and optionally a buffering agent and/or other excipients which further stabilize factor IX, as described, *supra*. The finished drug formulations are stable for at least six months and preferably up to 36 and 60 months; and can be stored at temperatures ranging from -100°C to 40°C, from -20°C to 37°C and from 2°C to 8°C. The concentrations of the excipients provide a combined osmolality of about 250 to 420 milliosmolal. Preferred formulations include factor IX concentrations ranging from about 0.1 to greater than 160 mg/mL (20 U/mL to greater than 56,000 U/mL); with sodium citrate as a buffering agent; some combination of mannitol, sucrose, arginine, and glycine as cryoprotectants and tonicity modifiers; and optionally small amounts of a chelator, such as EDTA (*ca.* 1 to 5 mM) and/or small amounts of polysorbate (0.005% to 0.02%). Other preferred formulations include factor IX (0.1 to greater than 160 mg/mL), glycine, a surfactant and/or buffer (*e.g.*, histidine) and/or a cryoprotectant (*e.g.* polysorbate).

Also provided by the invention are novel methods of administration of highly concentrated factor IX using both *intravenous* and *subcutaneous* routes, *e.g.*, an *intravenous* dose followed by a *subcutaneous* dose.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, factor IX includes both plasma derived and recombinantly or synthetically produced. Factor IX concentration is conveniently expressed as mg/mL or as U/mL, with 1 mg usually representing $> 150 \text{ U} \pm 100 \text{ U}$ or more. One Unit of activity is defined as the amount of factor IX clotting activity in one milliliter of normal human plasma. The specific activity is the ratio of clotting activity concentration to protein concentration, expressed as U/mg of protein. Patients with hemophilia generally have from < 1 to 25 % of the factor IX clotting factor as is found in normal human plasma.

As used herein, amounts specified are understood to be \pm about 10%, *e.g.*, about 50 mM includes $50 \text{ mM} \pm 5 \text{ mM}$; *e.g.*, 4% includes $4\% \pm 0.4\%$, *etc.*

As used herein, the term "tonicity modifier" includes agents which contribute to the osmolality of the solution. Examples of tonicity modifiers include, but are not limited to, amino acids such as arginine, histidine, and glycine, salts such as sodium chloride, potassium chloride, and sodium citrate, and saccharides such as sucrose, glucose, and mannitol, and the like.

The term "cryoprotectant" generally includes agents which provide stability to the protein from freezing-induced stresses; however, cryoprotectants may also provide general stability, for example for bulk drug formulations during storage from non-freezing-induced stresses. Exemplary cryoprotectants include polyols, and saccharides such as mannitol and sucrose, as well as surfactants such as polysorbate, or polyethyleneglycol, and the like. While preferred concentrations of cryoprotectant range from about 0.2 to 4% (weight/volume), relatively high concentrations, for example greater than 5%, are also suitable; the levels used are limited only by those customarily used in clinical practice. The upper concentration limits for bulk drug may be higher than for finished dosage, *e.g.*, greater than 5%. "Surfactants" generally include those agents which protect the protein from air/solution interface induced stresses and solution/surface induced stresses (*e.g.*, resulting in protein aggregation), and may include detergents such as polysorbate-80 (Tween), for example, about 0.005 to 1% (volume/volume), or polyethyleneglycol (PEG), such as PEG8000, for example. Optionally, relatively high concentrations, *e.g.*, up to 0.5%, are suitable for maintaining protein stability; however, the levels used in actual practice are customarily limited by clinical practice.

The term "buffering agent" encompasses those agents which maintain the solution pH in an acceptable range and may include histidine, phosphate (sodium or potassium), citrate (sodium or potassium), maleic acid, ammonium acetate, tris (tris (hydroxymethyl) aminomethane), diethanolamine, and the like. The upper concentration limits may be higher

for bulk protein than for finished dosage protein forms as is readily appreciated by one skilled in the art. For example, while buffer concentrations can range from several millimolar up to the upper limit of their solubility, *e.g.*, citrate, could be as high as 200 mM, one skilled in the art would also take into consideration both achieving and maintaining a physiologically appropriate concentration. Percentages are weight/volume when referring to solids dissolved in solution and volume/volume when referring to liquids mixed into solutions. For example, for sucrose, it is dry weight sucrose/volume of solution and for Tween, it is the volume of 100% stock/volume of solution. The term "isotonic with serum," 300 ± 50 milliosmolal, is meant to be a measure of osmolality of the solution prior to administration. Maintaining physiological osmolality is important for the dosage formulations to be injectable without prior dilution. However, for bulk formulations, much higher osmolalities can be effectively utilized as long as the solution is made isotonic prior to use. The term "excipients" includes pharmaceutically acceptable reagents to provide appropriate tonicity, cryoprotection of the protein, maintenance of pH, and proper conformation of the protein during storage so that substantial retention of biological activity and protein stability is maintained.

The following examples illustrate practice of the invention. These examples are for illustrative purposes only and are not intended in any way to limit the scope of the invention claimed. Example 1 describes the effect of calcium addition and the effect of pH on clotting activity. Example 2 describes the effects of specific buffering agents on the formation of high molecular weight aggregates (HMW). Example 3 illustrates the use of the invention for higher concentrations of factor IX. Example 4 illustrates the complexity of excipient interactions in stabilizing factor IX. Example 5 describes factor IX in various formulations relating to freeze/thaw stability. Example 6 describes the effects of long term storage, and Examples 7 and 8 illustrate highly concentrated forms and their use.

Example 1 - Effect of Calcium Ions

The preparation of recombinant factor IX has been described in USPN 4,770,999, Kaufman, *et al.* One suitable purification method is that described in Hrinda, *et al.*, Preclinical Studies of a Monoclonal Antibody - Purified Factor IX, Mononine™ Seminars in Hematology, 28(3):6 (July 1991). Other methods of preparation include the use of conformation-specific monoclonal antibodies as described by Tharakan, *et al.*, "Physical and biochemical properties of five commercial resins for immunoaffinity purification of factor IX." Journal of Chromatography 595:103-111 (1992); and by Liebman, *et al.*, "Immunoaffinity purification of factor IX (Christmas factor) by using conformation-specific antibodies directed against the factor IX-metal complex." Proc. Nat. Acad. Sci., USA 82:3879-3883 (1985); as well as

conventional chromatographic procedures, for example, as described by Hashimoto, *et al.*, "A Method for Systematic Purification from Bovine Plasma of Six Vitamin K-Dependent Coagulation Factors: Prothrombin, Factor X, Factor IX, Protein C, and Protein Z." *J. Biochem.* 97:1347-1355 (1985), and Bajaj, P. *et al.* *Prep. Biochem.* 11:397 (1981). "Large-scale preparation and biochemical characterization of a new high purity factor IX concentrate prepared by metal chelate affinity chromatography", P.A. Feldman *et. al.*, *Blood Coagulation and Fibrinolysis* 5:939-948 (1994). Yet another method of purification is described in USSN 08/472,823, filed June 7, 1995; and incorporated herein by reference.

A well characterized property of factor IX is its ability to bind Ca^{2+} ions. Structural studies indicate that Ca^{2+} binding may confer a more stable structure, reducing the probability of molecular motion ("Structure of the Metal-free γ -Carboxyglutamic Acid-rich Membrane Binding Region of Factor IX by Two-dimensional NMR Spectroscopy", S. J. Freedman, B. C. Furie, B. Furie, and J. D. Baleja, *J. Biol. Chem.* 270(14):7980-7987 (1995); "Structure of the Calcium Ion-Bound γ -Carboxyglutamic Acid-Rich Domain of Factor IX," S. J. Freedman, B. C. Furie, B. Furie, and J. D. Baleja, *Biochemistry* 34:12126-12137 (1994); "The Structure of a Ca^{2+} -Binding Epidermal Growth Factor-like Domain: Its Role in Protein-Protein Interactions", S. Rao, P. Handford, M. Mayhew, V. Knott, G. Brownlee, and D. Stuart, *Cell* 82:131-141 (1995); "Structure of Ca^{2+} Prothrombin Fragment 1 Including the Conformation of the Gla Domain", M. Soriano-Garcia, C.H. Park, A. Tulinsky, K. G. Ravichandran, and E. Skrzypczak-Jankun, *Biochem.* 28:6805-6810 (1989)). Presumably, less mobility accords a lower probability of molecular interaction, thereby reducing the probability of degrading processes. Surprisingly, this turns out not to be the case.

Samples are prepared in the formulations set forth in Table I below, at a recombinant factor IX protein concentration of ~ 0.5 mg/ml (100 U/ml) and an osmolality of 300 ± 50 milliosmolal. All samples contain a recombinant form of factor IX. To examine the potential utility of Ca^{2+} as a stabilizing agent, a set of samples was prepared in the formulations listed in Table 1. The formulation of sample A is the formulation used for commercially available plasma-derived lyophilized factor IX (Mononine[™]). All samples contain a recombinant form of factor IX.

Table 1
Sample Formulations

Sample	pH	Buffer (10 mM)	Salt (Tonicity Modifier)	Other Excipient
A	7.0	histidine	0.066 M NaCl (0.385%)	165 mM mannitol
B	7.0	histidine	260 mM glycine	29 mM sucrose
C	7.0	histidine	250 mM glycine, 5 mM Ca ²⁺	29 mM sucrose
D	7.5	tris	260 mM glycine	29 mM sucrose
E	7.5	tris	250 mM glycine, 5 mM Ca ²⁺	29 mM sucrose
F	7.5	diethanolamine	260 mM glycine	29 mM sucrose
G	7.5	diethanolamine	250 mM glycine, 5 mM Ca ²⁺	29 mM sucrose

Samples of factor IX in each formulation were stored at 4°C for 2.5 months. Samples were assayed for protein concentration and clotting activity. Factor IX activity is determined according to the method of Pittman, D., *et al.*, Blood 79:389-397 (1992) utilizing factor IX-deficient blood. The ratio of clotting activity to protein concentration, the specific activity, expressed as Units/mg of protein, is given in Table 2. An acceptable specific activity is one that is generally no more than 20% higher than the starting specific activity because an unusually high specific activity may indicate an activation-like event, which may have thrombotic implications.

Table 2
Factor IX Specific Activity

Sample	time zero	2.5 months
A	219.9	161.3
B	191.8	153.2
C	239.4	964.1
D	209.3	135.8
E	212.1	1956.9
F	190.1	123.5
G	217.3	2570.8

The samples containing calcium, *i.e.*, samples C, E, and G, have higher specific activities after 2.5 months of storage. This is due to the inclusion of Ca²⁺ and indicates that the factor IX has undergone a conversion to an activated-like molecule. Activated Factor IX is Factor IX that has been cleaved at residues R¹⁴⁵ - A¹⁴⁶ and R¹⁸⁰ - V¹⁸¹ and is then able to

catalyze clotting. Normally, Factor IX circulates as intact protein and is not converted to its activated form unless there is initiation of the clotting cascade. Injecting someone with activated rhFIX could have thrombotic implications. Therefore inclusion of Ca^{2+} at a concentration of 5 mM is destabilizing and is to be avoided.

5 **Example 2: Effects of Buffer Choice on HMW formation**

The average specific activity after eight months of 4°C storage of samples formulated in buffer/excipient combinations similar to and including those in Table 1, but without calcium, at pH 7.0 is 112.5 ± 10.5 U/mg, but at pH 7.5 is only 84.0 ± 22.1 U/mg, indicating subtle shifts in pH are significant for maintaining long-term factor IX stability.

10 Factor IX is prepared in a set of isotonic experimental formulations as summarized in Table 3, including several different excipient combinations for each buffering agent and some including less than 5 mM EDTA. Factor IX concentrations are approximately 1 mg/mL (average 161 U/mL). Samples are assayed for the amount of high molecular weight material (HMW) present and for clotting activity. The formation of significant (>3%) amounts of
15 HMW is undesirable and as indicative of physical degradation of factor IX with possible impact on product safety and efficacy. HMW is especially undesirable for *subcutaneous* administration as aggregated proteins are more immunogenic when given *subcutaneously*. Morein, B. and K. Simons, Vaccine 3:83. Subunit vaccines against enveloped viruses: virosomes, micelles, and other protein complexes (1985); and Antibodies: A laboratory manual, (page 100), E. Harlow
20 and D. Lane, Cold Spring Harbor Laboratory, 1988.

Table 3
Sample Formulations

Buffering Agent (10-15 mM)	Excipients
25 Phosphate (either sodium or potassium phosphate, pH 7.0)	arginine-HCl, sodium chloride, glycine, sucrose, mannitol, glucose
Citrate (sodium, pH 6.0-6.5)	sorbitol, glucose, glycine, sucrose, arginine-HCl
30 Ammonium Acetate (pH 6.5-7.0)	mannose, mannitol, sodium chloride, arginine-HCl
Maleic Acid (pH 6.5)	glycine, mannose

Table 4 shows the effects of the different buffering agents on HMW generation as measured by size exclusion chromatography (SEC-HPLC). Samples were stored at 30°C for six weeks. Table 4 gives the average increase expressed as (HMW/total protein x 100%) at six weeks minus that at time zero.

Table 4

Percent Increase HMW Generation

Buffering Agent	Avg. Increase (% of total)
Phosphate:	4.21
Citrate:	0.80
Ammonium Acetate:	3.42
Maleic Acid:	1.67

The citrate buffered samples had, on average, the smallest amount of HMW generated, regardless of the other excipients included. An appropriate buffer does not allow greater than a 2% increase.

Aggregates are commonly known to be more immunogenic than monomeric proteins and are generally not acceptable for an *intravenous* formulation. However, aggregates are even more undesirable for a *subcutaneous*, *intradermal* or *intramuscular* formulation, because these routes of administration are more likely to generate an immune response.

All samples are stored further for six months at 4°C and assayed for clotting activity. The average amount of activity remaining for samples containing the various saccharides varied greatly; sucrose-containing samples maintained an average 71% of the starting activity, mannitol 53%, glucose 52%, and mannose only 27%. Surprisingly, not all saccharides are equally effective at maintaining factor IX activity, despite the addition of other excipients.

Example 3: Stability at high concentration

Another set of formulations is prepared comprising higher concentrations of factor IX; samples are prepared in the formulations listed in Table 5 at a concentration of 8 mg/mL (2000 U/mL). All contain 15 mM sodium citrate and are buffered at pH 6.8, without surfactant. BG4 is slightly hypertonic, the rest are isotonic.

Table 5
Sample Formulations

BG1:	2% sucrose, 2% arginine-HCl, 1 mM EDTA
BG2:	4% sucrose, 1% glycine, 1 mM EDTA
BG3:	1.5% arginine-HCl, 1% glycine
BG4:	5% arginine-HCl, 1 mM EDTA
BG5:	4% sucrose, 1% glycine

Samples are stored in both glass vials and glass prefillable syringes for eight, ten and thirteen months at 4°C to determine whether the amount of air/solution interface or siliconized stopper/solution interface would impact the stability of the product. At all three time points no significant differences are seen by any stability assays between the vials and syringes. The results of several analytical methods are shown in Table 6. "Recovery of Activity" refers to the amount of clotting activity remaining in the sample expressed as a percentage of the amount of clotting activity present at "time zero", the start of the study. "HMW" is described, *supra*. "SDS-PAGE" is polyacrylamide gel electrophoresis; gels are scanned and bands quantified. Reversed phase HPLC is used to evaluate product heterogeneity and changes in peak ratios may indicate changes in the product, for example, oxidation of oligosaccharides.

Table 6

Sample	Recovery of Activity as % of control		HMW, as determined by SEC-HPLC		% full-length FIX, by SDS-PAGE		Reversed Phase HPLC ratio assay	
	8 mos.	13 mos.	8 mos.	13 mos.	8 mos.	10 mos.	8 mos.	13 mos.
BG-1	90%	92%	0.31	0.37	98.2	98.5	0.33	0.35
BG-2	78%	82%	0.33	0.32	98.0	98.4	0.32	0.37
BG-3	85%	82%	0.35	0.36	98.2	98.5	0.34	0.37
BG-4	83%	81%	0.25	0.28	98.6	99.1	0.33	0.36
BG-5	74%	81%	0.40	0.35	98.3	98.6	0.32	0.34

Even at this higher concentration of factor IX (8 mg/mL; 2000 U/mL) these formulations are effective. The increase in observed activity for certain samples at 13 months, relative to the control, is within the variability of the assay.

Example 4: Excipient Interactions

Another set of factor IX formulations, all containing citrate, is prepared as summarized in Table 7. All formulations are isotonic, contain factor IX at concentrations of 1 to 2 mg/mL (average 208 to 481 U/mL), use 10 mM to 15 mM sodium citrate as the pH buffering agent, and are adjusted to pH 6.8.

Table 7**Sample Formulations**

Major Excipient (range of concentration, wt/vol %)	Used in combination with:
mannitol (55 - 275 mM, 1-5%)	arginine-HCl, EDTA, glycine, Tween-80, sucrose, NaCl, KCl
arginine-HCl (47 - 237 mM, 1-5%)	mannitol, EDTA, sucrose, glycine, Tween-80, glucose
glycine (66 - 306 mM, 0.5-2.3%)	mannitol, arginine-HCl, glucose, Tween-80, EDTA
sucrose (29 - 234 mM, 1-8%)	mannitol, arginine-HCl, glycine, NaCl, EDTA, Tween-80
glucose (55 - 278 mM, 1-5%)	arginine-HCl, glycine, NaCl, KCl, EDTA
NaCl (100 mM, 0.58%)	sucrose, glucose, mannitol, EDTA
KCl (100 mM, 0.75%)	glucose, mannitol

Samples are stored at 4°C and assayed at several points in time. After eight months of 4°C storage, nine samples maintain ~ 100% of the clotting activity of the starting material. The formulations of these nine are shown in Table 8 (all include 15 mM sodium citrate, are pH 6.8, and isotonic).

Table 8

1	4% sucrose, 1.4% glycine, 0.005% Tween-80
2	1% mannitol, 2% arginine-HCl, 0.5% glycine
3	2.2% arginine-HCl, 0.75% glycine
4	3% mannitol, 1% glycine
5	3% mannitol, 1% glycine, 1 mM EDTA
6	3% mannitol, 1.5% arginine, 0.005% Tween-80
7	3.3% arginine-HCl
8	2% mannitol, 2% sucrose, 1.4% arginine
9	4% sucrose, 1.4% glycine, 1mM EDTA

Several formulations, containing similar excipients in similar ratios, nevertheless, surprisingly, do not maintain clotting activity nearly as well. For example, 2.3% glycine alone gave only 86%; and 4% sucrose, 2% arginine, both with and without Tween, and with and without EDTA gave 87-89% clotting activity.

Shown for the nine formulations of Table 8 are the results of other stability indicating assays. Specific activity is expressed as U/mg and an acceptable range is 200 to 350 U/mg. SEC-HMW is a measure of high molecular weight aggregates as determined by size-exclusion chromatography; less than 1% is preferred for a *subcutaneous*, *intradermal*, or *intramuscular* formulation. "C-terminal clips" is a measure of degradation species as determined by reversed phase chromatography; less than 1% is preferred.

TABLE 9

Sample	Recovery of Activity	Specific Activity	SEC HMW	C-Terminal Clips
1	$\geq 100\%$	262	0.24%	0.31%
2	$\geq 100\%$	256	0.25%	0.28%
3	$\geq 100\%$	255	0.27%	0.28%
4	$\geq 100\%$	262	0.26%	0.33%
5	$\geq 100\%$	272	0.23%	0.38%
6	$\geq 100\%$	263	0.22%	0.28%
7	$\geq 100\%$	258	0.24%	0.19%
8	$\geq 100\%$	251	0.20%	0.33%
9	$\geq 100\%$	251	0.20%	0.31%

Based on the preferred formulations set forth in Tables 8 and 9, two more preferred formulations include as follows: (both are buffered at pH 6.8 with 15 mM citrate and are isotonic), 3% mannitol, 1.5% arginine-HCl; and 3.3% arginine-HCl.

Example 5: Effects of Freeze/Thaw Cycle

Ideally, a similar formulation is utilized for bulk protein as is used for the finished dosage form. This demands that the same formulation that stabilizes factor IX from long-term storage stresses also be appropriate for stabilizing factor IX from the stresses normally encountered by bulk protein, such as freezing and thawing.

Samples are prepared in the formulations set forth in Table 10 below, at a protein concentration of ~ 2 mg/mL (500 U/mL) and an osmolality of 300 ± 50 milliosmolal. All include 10 mM sodium citrate, pH 6.8, and all are prepared both with and without 0.005% Tween-80 (polysorbate).

Table 10
Sample Formulations

A.	2.5% arginine-HCl, 2.2% sucrose
B.	1.8% glycine, 2% sucrose
C.	1.8% arginine-HCl, 2.4% mannitol
D.	2.2% glycine, 0.2% mannitol
E.	2.7% arginine-HCl, 0.8% mannitol
F.	2% arginine-HCl, 2% sucrose, 0.9% mannitol
G.	1.8% arginine-HCl, 2% mannitol, 0.8% sucrose

Samples of factor IX in each formulation were subjected to five freeze-thaw cycles to determine susceptibility to freezing-induced denaturation, which can result in formation of protein aggregates. A series of freeze-thaw cycles is a useful indication of a protein's susceptibility to increased aggregate formation as may be observed during freezing and long-term storage. Samples are assayed for the amount of HMW present. Samples with and without Tween-80 (0.005%) have minimal aggregation (less than 0.15% HMW increase).

Based on all the data herein, the following two formulations (expressed as ranges of components) are also preferred.

Formulation 1:		
sodium citrate	0.0075 M to 0.04 M	0.19% to 1% w/v
arginine (-HCl)	0.13 M to 0.16 M	2.8% to 3.3% w/v
sucrose	0 to 0.06 M	0 to 2% w/v
polysorbate-80	0 to 0.0000382 M	0 to 0.005% w/v
factor IX	600 to 56,000 Units/mL	0.1 to 160 mg/mL
Formulation 2:		
sodium citrate	0.0075 M to 0.04 M	0.19% to 1% w/v
arginine (-HCl)	0.06 M to 0.07 M	1.3 to 1.5%
sucrose	0 to 0.02 M	0 to 0.7%
mannitol	0.165 M	3%
polysorbate-80	0 to 0.0000382 M	0 to 0.005% w/v
factor IX	600 to 56,000 Units/mL	0.1 to 160 mg/mL

Example 6: Effect of Long Term Storage at 4°C

Factor IX is formulated at 2 mg/mL (500 U/mL) in 15 mM sodium citrate (0.38%), 0.16 M arginine (3.3%), pH 6.8 and stored for one year at 4°C. The recovery of activity is 95% and the % HMW is 0.32%. Factor IX is formulated at 2 mg/mL in 15 mM sodium citrate, 3% mannitol, 1.5% arginine, pH 6.8 and stored for one year at 4°C. The recovery of activity is 76% and the % HMW was 0.36%. The loss of activity is attributed to deamidation.

Factor IX is formulated at 2 mg/mL in 15 mM sodium citrate, 1% = 29 mM sucrose, 3% = 0.14 M arginine HCl and stored for one year at 4°C. The recovery of activity is 86% and % HMW is 0.27.

Example 7: Effects of High Protein Concentration and of Freeze-Thaw

Factor IX is formulated at 4000 U/mL, 8000 U/mL, 16,000 U/mL and greater than 30,000 U/mL (*i.e.*, 16 to greater than 120 mg/mL) in 10 mM histidine, 260 mM glycine, 1% sucrose, 0.005% Tween-80, pH 7.0. Factor IX is concentrated by centrifugal concentration in a Centricon-10 and by stir-cell concentration in an Amicon stir cell using a YM-10 membrane. Other methods used for concentrating proteins, especially those using membranes which retain and exclude species based on molecular weight, such as tangential flow filtration, can also be used. In addition, spray-drying can be used with no untoward effects.

Surprisingly, no detectable aggregated protein (HMW as determined by SEC-HPLC) is generated even at these extraordinarily high protein concentrations.

Samples are subsequently frozen and thawed repeatedly and surprisingly still maintain acceptable levels of HMW ($\leq 1\%$). This is surprising in light of the commercially available plasma-derived factor IX products such as Mononine™ and Alphanine™ (*supra* at page 3, lines 20-29), which frequently contain 10% or greater HMW even though the factor IX concentration is quite low. Such a high % HMW is unacceptable for *subcutaneous*, *intradermal*, or *intramuscular* administration because of the potential for immunogenicity.

Furthermore, when factor IX is formulated in the same formulation as Mononine™ and subjected to repeated cycles of freezing and thawing, significant amounts (~15%) HMW are generated. This data, taken with the data shown in Example 5, demonstrate the surprising and unpredictable effects of formulation on the stability of factor IX.

Example 8: Use of Highly Concentrated Factor IX

Highly concentrated factor IX is effective when administered *subcutaneously*, *intradermally* or *intramuscularly*. Utilizing a highly concentrated formulation of factor IX, *i.e.*, 4,000 U/mL to greater than 56,000 U/mL, a single site, low volume, *subcutaneous* injection is possible as is described below.

Three experimental groups were evaluated using factor IX at a concentration of 4,000 IU/ml in 260 mM glycine, 10 mM histidine, 29 mM (1 %) sucrose, and 0.005 % polysorbate. In Group I, dogs were given 200 U/kg (0.05 mL/kg) of factor IX *intravenously*. In Group II, dogs were given 200 U/kg (0.05 mL/kg) of factor IX *subcutaneously*. In Group III, dogs were given a factor IX *intravenous* priming dose of 50 U/kg (0.0125 mL/kg) followed 24 hours later by a 200 U/kg (0.05 mL/kg) *subcutaneous* dose. *Intravenous* factor IX produced a 240 % factor IX activity (where 100 % = pooled human plasma standard) within five minutes of injection which declined to 6.4 % by Day 5. *Subcutaneous* factor IX activity was 0.9 % at 5 minutes, 10 % at three hours and 5.8 % on Day 5. The combination of an *intravenous* loading dose followed 24 hours later by a *subcutaneous* dose resulted in a plasma factor IX activity of 25 % three hours after the *subcutaneous* dose and a factor IX activity of 9.1 % on Day 5 after the *subcutaneous* injection. The bioavailability of the *subcutaneous* dose was calculated as 43 %. *Subcutaneous* factor IX produces therapeutic levels of factor IX activity in less than three hours after administration. The combination dose of an *intravenous* with a *subcutaneous* dose provides immediate coagulant protection and improves the efficacy of the *subcutaneous* dose. Also, highly concentrated forms of factor IX can be formulated in the formulations described, *supra*, in Examples 1-5, and effectively used for administration.

While the present invention has been described in terms of specific methods, formulations, and compositions, it is understood that variations and modifications will occur to those skilled in the art upon consideration of the present invention.

Numerous modifications and variations in the invention as described in the above illustrative examples are expected to occur to those skilled in the art and, consequently, only such limitations as appear in the appended claims should be placed thereon. Accordingly, it is intended in the appended claims to cover all such equivalent variations which come within the scope of the invention as claimed.

WHAT IS CLAIMED:

1. A composition comprising factor IX and arginine.
2. The composition of claim 1, further comprising a buffering agent.
3. The composition of claim 1, further comprising a member selected from the group consisting of sucrose and mannitol.
4. The composition of claim 2, wherein said buffering agent is a member selected from the group consisting of citrate, maleic acid, ammonium acetate, phosphate, histidine, tris, and diethanolamine.
5. The composition of claim 1, further comprising a member selected from the group consisting of surfactant and chelating agent.
6. A composition comprising:
 - factor IX;
 - arginine;
 - a first member selected from the group consisting of sucrose and mannitol;
 - a second member selected from the group consisting of citrate, maleic acid, ammonium acetate, phosphate, histidine, tris, and diethanolamine; and
 - a third member selected from the group consisting of surfactant and chelating agent.
7. The composition of claim 1, wherein said arginine concentration is about 130 to 235 mM.
8. The composition of claim 1, wherein said arginine concentration is about 60 to 70 mM.
9. The composition of claim 7, wherein said arginine concentration is about 160 mM.
10. The composition of claim 3, wherein said first member is sucrose.
11. The composition of claim 10, wherein said sucrose concentration is about 3 to 60 mM.
12. The composition of claim 4, wherein said second member is citrate.
13. The composition of claim 12, wherein said citrate concentration is about 1 to 40 mM.
14. The composition of claim 13, wherein said citrate concentration is about 15 mM.

15. The composition of claim 6, wherein said third member is polysorbate.
16. The composition of claim 15, wherein said polysorbate concentration is about 0.005 to 1%.
17. The composition of claim 6, wherein said second member is citrate.
18. The composition of claim 6, wherein said first member is mannitol.
19. The composition of claim 6, wherein said arginine concentration is about 130 to 160 mM.
20. The composition of claim 6, wherein said arginine concentration is from about 60 to 70 mM.
21. The composition of claim 6, wherein said arginine concentration is about 130 mM to 160 mM, said sucrose concentration is about 0 to 60 mM, and said surfactant is about 0 to 0.005%.
22. The composition of claim 6, wherein said arginine concentration is about 130 to 160 mM, said sucrose concentration is about 0 to 60 mM, and said surfactant concentration is about 0 to 0.005%.
23. The composition of claim 6, wherein said arginine concentration is about 60 to 70 mM, said first member is about 0 to 20 mM sucrose and 165 mM mannitol, and said third member is 0 to 0.005% surfactant.
24. The composition of claim 6, wherein said arginine concentration is about 160 mM, said sucrose concentration is about 0 mM, said citrate concentration is about 15 mM, and said surfactant concentration is about 0%.
25. The composition of claim 6, wherein said arginine concentration is about 70 mM, said mannitol concentration is about 165 mM, said citrate concentration is about 15 mM, and said third member is 0%.
26. The composition of claim 6, wherein said factor IX concentration is about 0.1 to 160 mg/mL.

27. The composition of claim 26, wherein said factor IX concentration is about 2 to 100 mg/mL.
28. The composition of claim 27, wherein said factor IX concentration is about 0.1 to 10 mg/mL.
29. A composition comprising about:
 - 0.1 to 160 mg/mL factor IX,
 - 130 to 235 mM arginine, and
 - 7.5 to 40 mM citrate.
30. A composition comprising about:
 - 0.1 to 160 mg/mL factor IX,
 - 130 to 235 mM arginine,
 - 0 to 60 mM sucrose,
 - 0 to 0.005% polysorbate, and
 - 7.5 to 40 mM citrate.
31. A composition comprising about:
 - 0.1 to 160 mg/mL factor IX,
 - 66 to 90 mM arginine,
 - 110 to 165 mM mannitol, and
 - 7.5 to 40 mM citrate.
32. A composition comprising about:
 - 2 to 40 U/mL factor IX,
 - 130 to 235 mM arginine, and
 - 7.5 to 40 mM citrate.

33. A composition comprising about:
2 to 40 U/mL factor IX,
130 to 235 mM arginine,
0 to 60 mM sucrose,
0 to 0.005% polysorbate, and
7.5 to 40 mM citrate.
34. A composition comprising about:
2 to 40 U/mL factor IX,
66 to 90 mM arginine,
110 to 165 mM mannitol, and
7.5 to 40 mM citrate.
35. A composition comprising about:
0.1 to 160 mg/mL factor IX,
70 mM arginine,
165 mM mannitol, and
15 mM citrate.
36. A composition comprising about:
0.1 to 160 mg/mL factor IX,
160 mM arginine, and
15 mM citrate.
37. A composition comprising about 0.1 mg/mL to 160 mg/mL factor IX, glycine, a surfactant, and a member selected from the group consisting of a buffering agent and a cryoprotectant.
38. The composition of claim 37, wherein said buffering agent is a member selected from the group consisting of histidine, phosphate, tris, and diethanolamine.
39. The composition of claim 37, wherein said cryoprotectant is a member selected from

the group consisting of sucrose and mannitol.

40. The composition of claim 37, wherein said buffering agent is sodium phosphate, wherein said cryoprotectant is sucrose, and wherein said surfactant is polysorbate.

41. The composition of claim 37, wherein said glycine concentration is about 0.1 to 0.3 M.

42. The composition of claim 41, wherein said glycine concentration is about 0.26 M.

43. The composition of claim 37, wherein said buffering agent is about 5 to 30 mM histidine.

44. The composition of claim 43, wherein said histidine concentration is about 10 mM.

45. The composition of claim 37, wherein said cryoprotectant is about 0.5 to 2% sucrose.

46. The composition of claim 45, wherein said sucrose concentration is about 1%.

47. The composition of claim 37, wherein said surfactant is about 0.005 to 0.05% polysorbate.

48. The composition of claim 47, wherein said polysorbate concentration is about 0.005%.

49. A composition comprising about 0.1 mg/mL to 160 mg/mL factor IX, 10 mM histidine, 0.26 M glycine, 1% sucrose, and 0.005% polysorbate.

50. A method for increasing factor IX concentration comprising administration of a composition of claim 1.

51. A method for increasing factor IX concentration comprising administration of a composition of claim 6.

52. A method for increasing factor IX concentration comprising administration of a composition of claim 35.

53. A method for increasing factor IX concentration comprising administration of a composition of claim 37.

54. A method for increasing factor IX concentration comprising administration of a composition of claim 49.

55. A method for increasing factor IX concentration comprising administration of a composition of claim 6, both *intravenously* and *subcutaneously*.
56. A method for increasing factor IX concentration comprising administration of a composition of claim 26, both *intravenously* and *subcutaneously*.
57. A method for increasing factor IX concentration comprising administration of a composition of claim 49, both *intravenously* and *subcutaneously*.

INTERNATIONAL SEARCH REPORT

Inter national Application No
PCT/US 97/00747

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/48 A61K47/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 28954 A (GENETICS INSTITUTE, INC.) 2 November 1995 see page 3, line 4 - page 4, line 19; claims	37-49, 53,54,57
A	--- EP 0 317 376 A (CENTRE REGIONAL DE TRANSFUSION SANGUINE DE LILLE) 24 May 1989 see page 3, column 4, line 36 - line 45; claims 12-14,17	1-36, 50-52, 55,56
A	--- WO 95 26750 A (PHARMACIA AB) 12 October 1995 see page 6, line 30 - page 7, line 13 see page 9, line 36 - page 10, line 3 --- -/--	1-36, 50-52, 55,56

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *A* document member of the same patent family

Date of the actual completion of the international search

20 May 1997

Date of mailing of the international search report

24.05.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Ryckebosch, A

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/00747

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>EP 0 430 200 A (BEHRINGWERKE AG) 5 June 1991</p> <p>see claims 1,5-9</p> <p>-----</p>	<p>1-36, 50-52, 55,56</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/00747

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 50-57
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/US 97/00747

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9528954 A	02-11-95	AU 2207595 A CA 2182200 A EP 0758248 A	16-11-95 02-11-95 19-02-97

EP 317376 A	24-05-89	FR 2622108 A FR 2631830 A DE 3877529 A DE 3877529 T ES 2045167 T JP 1207239 A NO 179997 B US 5457181 A	28-04-89 01-12-89 25-02-93 07-11-96 16-01-94 21-08-89 21-10-96 10-10-95

WO 9526750 A	12-10-95	AU 2156095 A CA 2186126 A EP 0772452 A FI 963871 A NO 964081 A PL 316618 A ZA 9502272 A	23-10-95 12-10-95 14-05-97 27-09-96 29-11-96 20-01-97 12-12-95

EP 430200 A	05-06-91	DE 3939346 A AT 106746 T AU 657466 B AU 6702990 A CA 2030988 A DE 59006032 D ES 2057339 T IE 64080 B JP 3170437 A	06-06-91 15-06-94 16-03-95 06-06-91 30-05-91 14-07-94 16-10-94 12-07-95 24-07-91

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